UltraRapid Communication

Angiopoietin-1 Promotes Cardiac and Skeletal Myocyte Survival Through Integrins

Susan M. Dallabrida, Nesreen Ismail, Julianne R. Oberle, Blanca E. Himes, Maria A. Rupnick

Abstract—Cardiac myocyte loss, regardless of insult, can trigger compensatory myocardial remodeling leading to heart failure. Identifying mediators of cardiac myocyte survival may advance clinical efforts toward myocardial preservation. Angiopoietin-1 limits ischemia-induced cardiac injury. This benefit is ascribed to angiogenesis because the receptor, tie2, is largely endothelial-specific. We propose that direct, non-tie2 interactions of angiopoietin-1 on cardiac myocytes contribute to this cardioprotection. We found that mouse C2C12 skeletal myocytes lack tie2, yet dose-dependently adhered to angiopoietin-1 and angiopoietin-2 similarly to laminin, fibronectin, vitronectin, and more than to collagen-I, -III, and -IV. Adhesion was divalent cation-mediated (Mn²⁺, Ca²⁺, not Mg²⁺), blocked with EDTA/EGTA, RGD-based peptides, and select integrin subunit antibodies. Similar findings were obtained with human skeletal myocytes (HSMs) and freshly isolated rat neonatal cardiac myocytes (NCMs). Furthermore, angiopoietin-1 conferred significant survival advantage exceeding that of most cell matrices, which was not fully explained by differences in cell adhesion. Angiopoietin-1 promoted survival of serum-starved C2C12, HSM, and NCM (MTT, trypan blue) and prevented taxol-induced apoptosis (caspase-3). Immobilized and soluble angiopoietin-1 phosphorylated AktS473 and MAPKp42/44 (not FAKY397) in C2C12 more than in endothelial cells and more than did angiopoietin-2 or cell matrices. EDTA, RGD-based peptides, and some integrin antibodies blocked these responses. Angiopoietin-1 activated HSM and NCM AktS473 and MAPKp42/44 survival pathways. We propose that this novel function contributes to developmental and cardioprotective actions of angiopoietin-1 presently attributed to vascular effects alone. Angiopoietin-1 may prove therapeutically valuable in cardiac remodeling by supporting myocyte viability and preserving pump function. The full text of this article is available online at http://circres.ahajournals.org. (Circ Res. 2005;96:e8-e24.)

Key Words: angiopoietin-1 ■ angiopoietin-2 ■ cardiac myocytes ■ adhesion molecules ■ myocyte apoptosis ■ skeletal myocytes

There is growing consensus that cardiomyocyte (CM) apoptosis contributes to many cardiac diseases (eg, ischemia, infarction, hypertension, myocarditis, transplant rejection, and heart failure). Research efforts are directed at defining the incidence of CM death, the contributions to cardiac dysfunction, and the consequences of inhibiting apoptosis. Incentive is based on the rationale that CM loss reduces contractile mass of the heart and may be a preventable catalyst of heart failure. In support of this concept, low levels of CM apoptosis (23 CM/10⁵ nuclei) cause lethal cardiomyopathy in mice. CM apoptosis rates are higher in cardiomyopathy patients (80 to 250 CM/10⁵ nuclei) compared with healthy hearts (1 to 10 CM/10⁵ nuclei). Further, ischemic preconditioning upregulates bcl-2, a cytoprotective protein, and is linked to reduced apoptosis. Despite amassing experimental and clinical evidence, mechanisms and signaling pathways in CM apoptosis are largely unexplored. Identifying regulatory mediators may lead to novel therapies to preserve myocardial function after injury. Toward this goal, we introduce a novel function for angiopoietin-1 as a direct cardiac myocyte survival factor.

Angiopoietin-1 (Ang1) and angiopoietin-2 (Ang2) are secreted proteins that bind the tie2 receptor, which is largely limited to endothelium. Angiopoietins are most noted as regulators of vascular maturation. Ang1 associates with matrix and acts locally, whereas Ang2 freely diffuses. Ang1 promotes endothelial cell survival and increases mural cell contact and matrix contacts with vessels to establish quiescence and promote maturation. Ang2 often favors vessel destabilization allowing angiogenesis or regression at high concentrations acts as a weak agonist, and is an agonist on lymphatic endothelium. A role for angiopoietins in cardiovascular health and disease is emerging. Ang1 and tie2 knockouts result in...
embryonic lethal cardiac defects, including impairments in vascular maturation and in endocardial and trabeculae formations. A similar phenotype is produced by transgenic overexpression of Ang2, a competitive Ang1 antagonist. Postnatally, as the heart transitions from extensive neonatal remodeling to limited adult remodeling, Ang1 levels increase. The endothelial-specific tie2 receptor is constitutively activated in adult hearts, suggesting Ang1 may serve a maintenance function for the vasculature.

The Ang/tie2 system is also implicated in cardiac remodeling under pathological conditions. Expressions of Ang2 increase after myocardial infarction and plasma Ang2 levels are higher in patients with heart failure. In contrast, Ang1 expressions are reduced in hypoxic myocardium. Ang1 overexpression in mice, rats, and rabbits with acute myocardial infarcts reduced infarct sizes and preserved ejection fractions. These benefits were ascribed solely to the role of Ang1 in angiogenesis because the tie2 receptor is endothelial-specific. However, fibroblasts were recently shown to adhere to Ang1 and Ang2 via integrins, raising the possibility that other nonendothelial cell types, such as CM may directly interact with angiopoietins.

Integrins are cell surface adhesion receptors composed of α and β subunits, which combine to form at least 24 heterodimers with different, although often overlapping, ligands and signaling properties. Integrins are also regulated by dynamic spatial and temporal expression patterns and subunit isoforms. For example, integrin adhesion, expression, and activation shift during cardiac development, hypertrophy, infarct, and failure. Information traffic via integrins is bidirectional, enabling cells to interact with the extracellular matrix (ECM)/environment. Integrins thereby mediate numerous vital CM activities such as cell shape, adhesion, apoptosis, anoikis, hypertrophy, survival, differentiation, contraction, and conduction.

In this study, we present the first evidence that cardiac and skeletal myocytes adhere to Ang1 and Ang2 via integrins. We show that Ang1 markedly promotes CM survival under stress, and Ang1 protects CM from apoptosis. This raises the possibility that the cardioprotective benefits of Ang1 overexpressed in ischemic hearts are due, in part, to direct interactions between Ang1 and CM, mediated via integrins. If so, angiopoietin regulation may serve as a novel target for preserving myocyte viability after cardiac insults, impeding heart failure development.

Materials and Methods

Details of the following methods can be found in the Expanded Materials and Methods section of the online data supplement available at http://circres.ahajournals.org.

Cell Culture

We chose to use the C2C12 cell line (American Tissue Culture Collection) because these transformed mouse skeletal myoblasts, once differentiated, have features of CM such as expression of cardiac isoforms of contractile proteins and well-organized myofibrils. Further, using a cell line eliminates the possibility of contaminating cells found in primary cultures. C2C12 cells were grown in high-glucose (4.5 g/L) DMEM (HG-DMEM) (Gibco) supplemented with 10% heat-inactivated fetal calf serum (FCS) (HyClone)/0.01 mol/L HEPES (Gibco)/L-glutamine- penicillin G-streptomyein sulfate (GPS) (Gibco) (37°C, 5% CO2). For adhesion, survival, and caspase-3 assays, C2C12 myoblasts were differentiated to myocytes by confluence. Cells that were 4 to 7 days postconfluence were used. Microvascular endothelial Ms1 cells were cultured in low glucose (1.0 g/L) DMEM (LG-DMEM) supplemented with 10% FCS/GPS (37°C, 10% CO2). Human skeletal muscle primary myoblasts (Cambrex) were grown in Clonetics SkGM BulletKit Medium plus growth factors (37°C, 5% CO2) and differentiated to myocytes by confluence and growth factor withdrawal as per manufacturer’s instructions.

Cardiac Myocyte Isolation

Cardiac myocytes were isolated from the ventricles of Sprague-Dawley P2 rat pups (Charles River Laboratories, Wilmington, Mass) following published procedures. Briefly, ventricles were minced, digested in 0.6 mg/mL trypsin/EDTA at 4°C overnight, then 1 mg/mL collagenase for 30 minutes 37°C. and preplated. Myocytes were collected, rinsed, and grown in LG-DMEM/2% FCS.

RT-PCR

Cells were immersed in RNALater (Ambion). Total RNA was purified using RNeasy mini kits (Qiagen). cDNA was made using Advantage RT-for-PCR kits (Clontech) with RNA (1 μg). PCR with tie1, tie2, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Invitrogen) primers were performed as we have described.

Cell Adhesion Assay

Assays were conducted as we and others have described with some modifications. Human recombinant Ang1 and Ang2 (R&D Systems) were dissolved in 0.1% bovine serum albumin (BSA), fraction V phosphate-buffered saline (PBS) (Fisher), and then further diluted in PBS at 0 to 400 nmol/L. BSA controls contained comparable amounts of 0.1% BSA/PBS diluted in PBS. Ang1, Ang2, and BSA control solutions were used to coat wells in 96-well flat-bottom-tissue culture plates (room temperature, 1 hour). Wells were also coated with human fibronectin, vitronectin, collagen I, collagen III, and mouse laminin and collagen IV as per the manufacturers’ instructions. Wells were blocked for at least 30 minutes with 0.5% heat-inactivated BSA (10 minutes, 80°C) with PBS, rinsed three times with PBS, and prepared cells were added. C2C12 myocytes, primary rat neonatal cardiac myocytes (NCMs), and primary human skeletal myocytes (HSMS) were detached with trypsin/EDTA, rinsed in serum-free medium, plated onto immobilized proteins, and incubated for 40 minutes. Wells were rinsed, attached cells fixed, toluidine blue-stained, solubilized, and absorbances measured (650 nm). Values were corrected for background myocyte adhesion to BSA wells. For some assays, adhesion was challenged with EDTA, EGTA, GRGESP (RGE), or GRGDSP (RGD) peptides, or adhesion-blocking integrin subunit antibodies. Assays were also done in which divalent cations were depleted and replaced (CaCl2, MgCl2, MnCl2, or no divalent cations).

Cell Survival Assays

A trypan blue assay and an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (Sigma) assay with improved sensitivity were used to measure myocyte viability compared with the various extracellular matrices. C2C12, HSM, and NCM were prepared and 96-well plates were coated as described (adhesion assay) using 200 nmol/L solutions to coat the plate surfaces.

Caspase-3 Assay

Apoptosis was measured in C2C12 myocytes, NCMs, and HSMS using a Caspase Colorimetric Kit (Promega) as per manufacturer’s instructions. Myocytes were prepared and 96-well plates were coated as described (adhesion assay) using 200 nmol/L solutions to coat the plate surfaces.
Cell Signaling
Phosphorylation of Akt (protein kinase B) serine 473 (pAktS473), mitogen-activated protein kinase (MAPK) p42/44 (ERK 1/ERK 2) threonine 202/tyrosine 204 (pMAPKp42(T202)/44(Y204)), and focal adhesion kinase (FAK) tyrosine 397 (pFAK Y397) were measured in myocytes (C2C12, HSM, NCM) incubated either on immobilized or in soluble molecules (200 nmol/L Ang1, Ang2, laminin, fibronectin, vitronectin, collagens-I, -III, and -IV, BSA) or on the plate alone (unmodified tissue culture wells), and examined by Western blotting. We conducted two types of soluble studies in which activation of Akt and MAPKp42/44 were measured. In one, we added 200 nmol/L soluble molecules to cells (C2C12, Ms1) in suspension. In the other, we plated cells (C2C12, HSM, Ms1) onto tissue culture plates, serum-starved cells, added wortmannin to some wells, then added (3.6 or 200 nmol/L) soluble molecules, and made protein lysates.

Results
Skeletal and Cardiac Myocytes Adhere to Ang1 and Ang2
C2C12 mouse skeletal myocytes (Figure 1A), human skeletal myocytes (HSMs, Figure 1B), and rat neonatal cardiac myocytes (NCMs, Figure 1C) all adhered to immobilized Ang1 and Ang2 in a concentration-dependent manner. In contrast, undifferentiated C2C12 myoblasts did not attach to either angiopoietin (data not shown). The number of myocytes adhering to Ang1 versus Ang2 was similar among the cell types.

We compared myocyte adhesion to angiopoietins to six ECM/basement membrane (BM) components prominent in the heart (Figure 1). C2C12 myocyte adhesion curves for Ang1, Ang2, laminin, fibronectin, and vitronectin were similar, whereas little adhesion occurred on collagen-I, -III, or -IV (Figure 1A). In contrast, HSMs adhered to all the matrix components and at lower surface coating concentrations than to Ang1 or Ang2 (Figure 1B). However, when the plates were coated with 200 nmol/L of each molecule, the number of adherent HSMs plateaued and was similar on all the surfaces. At that concentration, NCM also adhered to Ang1 and Ang2 similarly to most other matrix molecules, with the exception of superior adhesion to collagen–IV (Figure 1C). The concentration for maximum adhesion of myocytes to Ang1 and Ang2 is equal to or less than that for fibroblasts to Ang1/
Ang2 and for endothelial cell (EC) to angiopoietin-like protein-3 (Angptl3), which does not bind tie2.

Based on these findings, 200 nmol/L coating concentrations were used for the matrices in subsequent assays. This enabled the functional effects of each matrix to be assessed at comparable cell densities. The exceptions were poor adhesion of C2C12 to the collagens and greater adhesions of NCM to collagen-I and -IV.

Our data shows that mouse, rat, and human myocytes (primary cells, cell lines) interact with human Ang1 and Ang2. This likely reflects the highly conserved amino acid sequence. Ang1 homology for human versus mouse is 97% and for human versus rat is 96%. Human Ang2 is 85% identical to mouse and 86% identical to rat sequence.

Myocyte Adhesion to Ang1/Ang2 Does Not Involve the Tie2 Receptor and Is Inhibited by RGD and EDTA

We conducted RT-PCR of tie2, tie1 (related receptor that does not bind angiopoietins), and GAPDH (housekeeping gene) using Ms1 (mouse microvascular endothelial cells), HMVECs (human microvascular endothelial cells), and rat left ventricle (LV) tissue as positive controls (tie2, tie1). C2C12 myocytes, HSM, and NCM did not express mRNA for tie2 or tie1 (Figure 2A). To determine whether tie2 expression was conditional, we conducted RT-PCR and real-time PCR for tie2 and GAPDH with C2C12 myocytes that were cultured in full and serum-free medium. Tie2 mRNA was not detectable under any condition used in our assays, whereas GAPDH mRNAs were abundant (data not shown). Thus, angiopoietin/myocyte interactions do not involve tie2.

We measured Ang1 and Ang2 mRNA expressions in C2C12 myocytes, NCM, and HSM using mouse, rat, and human heart as positive controls. Skeletal and cardiac myocytes express Ang1 mRNAs (Figure 2A). Only C2C12 myocytes express Ang2 transcripts.

We propose that integrins mediate myocyte interactions with Ang1 and Ang2. Integrin adhesions require divalent cations with 3 to 5 divalent cation binding sites per integrin heterodimer. Integrins also require an exposed aspartic (D) or glutamic acid (E). There are different D-based (LDV, RTD, REDV, KRLDG) and E-based (LRE) motifs, but RGD-based motifs are most common. Requiring divalent cations and antagonism by RGD peptides would support a role for integrins in mediating myocyte-angiopoietin interactions.
C2C12 myocyte adhesion to Ang1 and Ang2 were significantly blocked by RGD-based peptides (74.1±7.5%, 89.2±10.1%), EDTA (96.0±1.5%, 95.8±3.7%), and EGTA (95.5±5.8%, 91.8±3.0%) (Figure 2B). EDTA nonspecifically chelates divalent cations, whereas EGTA has a higher affinity for Ca\(^{2+}\) than Mg\(^{2+}\). To define the divalent cations mediating C2C12 myocyte adhesion to Ang1 and Ang2, we examined the effects of Ca\(^{2+}\), Mg\(^{2+}\), Mn\(^{2+}\), and no divalent cations.\(^4\),\(^5\) Mn\(^{2+}\) and Ca\(^{2+}\) supported adhesion to Ang1 and Ang2, but Mg\(^{2+}\) did not (Figure 2C). Ca\(^{2+}\) occupies divalent cation sites in many integrins,\(^5\) Mn\(^{2+}\) activates integrins, increasing the kinetic “on” rate.\(^4\),\(^5\) A determinant of integrin high-affinity binding (firm adhesion) to a ligand is activation from a low to high affinity state, involving a conformational change.\(^50\) Overall, these findings suggest that myocyte adhesion to Ang1 and Ang2 is integrin-mediated.

### Integrins Mediate Skeletal and Cardiac Myocyte Adhesion to Ang1 and Ang2

To assess whether integrins mediate the attachment of C2C12 myocytes, NCM, and HSM to Ang1 and Ang2, we conducted adhesion assays using adhesion-blocking integrin antibodies. C2C12 myocyte adhesion to Ang1 was inhibited by \(\alpha_\alpha\) (93.5±5.6%), \(\beta_\beta\) (53.9±13.6%), \(\beta_\beta\) (44.1±11.5%), \(\alpha_\alpha\) (39.9±4.1%), and \(\alpha_\alpha\) (35.1±2.7%), but not \(\alpha_\beta\) or \(\alpha_\beta\) antibodies (Figure 3A). Adhesion to Ang2 was blocked by \(\beta_\beta\) (71.1±2.5%), \(\beta_\beta\) (66.7±1.8%), \(\alpha_\alpha\) (61.3±1.7%), \(\alpha_\alpha\) (39.3±8.8%), and \(\alpha_\alpha\) (36.9±5.9%), but not \(\alpha_\beta\) or \(\alpha_\beta\) antibodies (Figure 3A). Integrin subunits \(\alpha_\alpha\) and \(\beta_\beta\) appear to be key mediators of C2C12 myocyte adhesion. Subunit \(\alpha_\alpha\) can pair with \(\beta_\beta\) or \(\beta_\beta\). Currently, no anti-mouse adhesion-blocking \(\beta_\beta\) integrin subunit antibodies are commercially available, so we were unable to further analyze \(\alpha_\alpha\) involvement using this method. Integrin subunit \(\beta_\beta\) only complexes with \(\alpha_\alpha\), except for platelets where \(\beta_\beta\) complexes with \(\alpha_\alpha\). In line with our results that \(\beta_\beta\) mediates adhesion, \(\alpha_\alpha\) (Figures 3B). NCM adhesion to Ang1 was inhibited by \(\beta_\beta\) (81.3±3.8%) and \(\beta_\beta\) (67.1±14.4%), but not \(\alpha_\alpha\) antibody (Figure 3B). NCM adhesion to Ang2 was also blocked by \(\beta_\beta\) (67.5±7.9%) and \(\beta_\beta\) (91.9±6.9%), but not \(\alpha_\alpha\) antibody (Figure 3B). Overall, these findings suggest that myocyte adhesion to Ang1 and Ang2 is integrin-mediated.

Figure 3. Integrins mediate skeletal and cardiac myocyte adhesion to Ang1 and Ang2. C2C12 myocytes (A), NCMs (B), or HSMs (C) were preincubated with PBS (control) or 50 μg/mL of different integrin antibodies diluted in PBS (n=3/group per study; studies done in duplicate), and adhesion to Ang1 and Ang2 was quantified using toluidine blue absorbances. Antibodies reducing cell adhesion to Ang1 or Ang2 are indicated (*\(P\leq0.03\) Ang1; #\(P\leq0.03\) Ang2).
Ang1 had increased survival compared with the other conditions (Figure 4B and 4C).

To assess if Ang1 enables cardiac myocyte survival, we conducted trypan blue and MTT-based viability/survival assays. Myocytes were incubated in serum-free media. Ang1 significantly induced C2C12 myocyte phosphorylation of Akt<sub>ser473</sub> (Figure 6A) and MAPK<sub>p42/44</sub> (Figure 6B) more than any other matrix (*P<0.0001 Ang1 versus other matrix), whereas FAK activation was not affected (Figure 6C). Ang1 also activated Akt<sub>ser473</sub> (Figure 7A) and MAPK<sub>p42/44</sub> (Figure 7B) in HSMs more than all other conditions (*P<0.0001). In NCMs as well, Ang1 promoted phosphorylation of Akt<sub>ser473</sub> (Figure 8A) and MAPK<sub>p42/44</sub> (Figure 8B) more than all other ECM (*P<0.005), but did not alter FAK activation (Figure 8C). The ability of Ang1 to increase Akt and MAPK<sub>p42/44</sub> phosphorylation in myocytes suggests a novel mechanism that may have therapeutic value in preserving CM viability and function after injury.

**Ang1-Mediated Myocyte Activation of Akt and MAPK<sub>p42/44</sub> Signals in Skeletal and Cardiac Myocytes**

We used Western blotting to determine whether myocyte interactions with Ang1 and Ang2 activate Akt, MAPK<sub>p42/44</sub>, and FAK signals, because these pathways increase survival and prevent apoptosis of CM. Comparisons were made among myocytes plated on the various immobilized matrices or suspended (Sus) in serum free media. Ang1 significantly induced C2C12 myocyte phosphorylation of Akt<sub>ser473</sub> (Figure 6A) and MAPK<sub>p42/44</sub> (Figure 6B) more than any other matrix (*P<0.0001 Ang1 versus other matrix), whereas FAK activation was not affected (Figure 6C). Ang1 also activated Akt<sub>ser473</sub> (Figure 7A) and MAPK<sub>p42/44</sub> (Figure 7B) in HSMs more than all other conditions (*P<0.0001). In NCMs as well, Ang1 promoted phosphorylation of Akt<sub>ser473</sub> (Figure 8A) and MAPK<sub>p42/44</sub> (Figure 8B) more than all other ECM (*P<0.005), but did not alter FAK activation (Figure 8C). The ability of Ang1 to increase Akt and MAPK<sub>p42/44</sub> phosphorylation in myocytes suggests a novel mechanism that may have therapeutic value in preserving CM viability and function after injury.

**Ang1-Mediated Myocyte Activation of Akt and MAPK Occurs via Integrins**

To assess whether Ang1 activation of myocyte Akt and MAPK<sub>p42/44</sub> occurs via integrins, we preincubated C2C12 myocytes with EDTA, GRGDSP or GRGESP peptides, integrin antibodies (α<sub>i</sub>, α<sub>v</sub>, β<sub>3</sub>), or PBS, and then plated them on 200 nmol/L immobilized Ang1 or vitronectin. We chose vitronectin for comparison because it induced the second highest Akt phosphorylation after Ang1 (Figure 6A). Furthermore, cell interactions with vitronectin via integrins can be disrupted with RGD-based peptides and EDTA. Again, suspended myocytes

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**Table: Skeletal and Cardiac Myocyte Viability on Different ECMs**

<table>
<thead>
<tr>
<th>Matrix</th>
<th>% Viable C2C12 Mean±SD</th>
<th>% Viable HSMs Mean±SD</th>
<th>% Viable NCMs Mean±SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ang1</td>
<td>97.87±1.02</td>
<td>94.70±0.3</td>
<td>82.40±0.54</td>
</tr>
<tr>
<td>Ang2</td>
<td>92.23±1.28*</td>
<td>95.95±0.92#</td>
<td>69.59±0.88†</td>
</tr>
<tr>
<td>Collagen I</td>
<td>91.53±1.47†</td>
<td>96.40±0.79</td>
<td>76.40±0.56†</td>
</tr>
<tr>
<td>Collagen III</td>
<td>94.39±0.62**</td>
<td>90.62±1.82*</td>
<td>76.68±0.87***</td>
</tr>
<tr>
<td>Collagen IV</td>
<td>88.83±1.15***</td>
<td>85.87±0.77†</td>
<td>76.23±1.02***</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>86.34±0.66†</td>
<td>83.84±1.76†</td>
<td>68.72±0.61†</td>
</tr>
<tr>
<td>Laminin</td>
<td>88.99±0.77†</td>
<td>87.23±0.93†</td>
<td>75.43±0.84‡</td>
</tr>
<tr>
<td>Vitronectin</td>
<td>88.63±1.06***</td>
<td>81.39±0.89†</td>
<td>75.89±1.50‡</td>
</tr>
<tr>
<td>Plate</td>
<td>79.32±0.84†</td>
<td>89.41±2.52**</td>
<td>75.08±0.99***</td>
</tr>
<tr>
<td>BSA</td>
<td>65.11±0.49‡</td>
<td>77.09±0.76†</td>
<td>48.33±1.02‡</td>
</tr>
</tbody>
</table>

*P<0.0004; **P<0.001; ***P<0.0002; †P<0.0000002; ‡P<0.02.
were used as a negative control. EDTA significantly reduced myocyte Akt (Figure 9A and 9B) and MAPKp42/44 (Figure 9C and 9D) activation when incubated on Ang1 (Figure 9A, Akt; Figure 9C, MAPK) or vitronectin (Figure 9B, Akt; Figure 9D, MAPK) (*P ≤ 0.002). GRGDSP peptide was consistently more effective at blocking Ang1- (Figure 9A, Akt; Figure 9C, MAPK) and vitronectin- (Figure 9B, Akt; Figure 9D, MAPK) induced cell signaling, than GRGESP peptide (#P ≤ 0.05). GRGESP peptide reduced myocyte Akt and MAPKp42/44 activation, which may reflect nonspecific interference with integrin adhesion.53

Figure 4. Ang1 increases skeletal and cardiac myocyte survival. MTT-based survival studies were conducted with (A through C) C2C12 myocytes or (D and E) NCMs. Myocytes were plated in serum-free media (n=3/group per study; studies done in duplicate) on 200 nmol/L (A and B, D and E) Ang1, Ang2, unmodified tissue culture wells (plate), or BSA for 1 to 7 days or (C) on Ang1, fibronectin (FN), or laminin (lam) for 1 day. MTT was quantified by absorbance at 570 nm. A and D, Photomicrographs were taken (bar=100 microns). Increases in C2C12 myocyte survival on Ang1 vs (B) BSA (*P ≤ 0.001), plate (*P ≤ 0.001), and Ang2 (*P ≤ 0.005); (C) fibronectin (*P = 0.003) and laminin (**P = 0.0001) are indicated. B, Myocyte viability on Ang2 exceeded BSA and plate (#P ≤ 0.04) on day 1. E, Increases in NCM survival on Ang1 vs BSA (*P = 0.004), plate (*P = 0.0009), and Ang2 (*P ≤ 0.0002) are indicated.
Ang1-induced Akt phosphorylation was significantly blocked by anti-β1 (*P=0.001), with a trend for anti-α6 (P=0.06), and was not effected by anti-α5 (Figure 9E). Ang1 activation of MAPKp42/44 was blocked by anti-β1 more than anti-α5 (*P=0.001), but not anti-α5 (Figure 9F). Thus, antibodies to anti-α5 did not affect myocyte adhesion (Figure 3A) or signaling, but antibodies to β1 and α6 reduced both myocyte adhesion (Figure 3A) and signaling. This data demonstrates that Ang1-induced activation of myocyte Akt and MAPKp42/44 is integrin-mediated.

Soluble Ang1 Activates Myocyte Akt and MAPKp42/44 Signaling

To determine whether Ang1-induced phosphorylation of Akt and MAPKp42/44 required cell adhesion, we placed C2C12 myocytes and Ms1 microvascular endothelial cells in suspension in serum-free medium and added 200 nmol/L Ang1, Ang2, fibronectin, laminin, vitronectin, or PBS. Western blotting showed that Ang1 markedly phosphorylated Akt (Figure 10A) and MAPKp42/44 (Figure 10B) in both cell types considerably more than did the other soluble conditions (*P≤0.001 Ang1 versus other conditions). Activation was greater in myocytes (65.7-fold Akt; 3.5 fold MAPK) than endothelial cells (3.4-fold Akt; 2.1-fold MAPK). Thus, Ang1 potently activated cytoprotective signaling pathways in myocytes in a non-tie2, cell adhesion–independent manner.

Ang1 has been shown in several reports to activate endothelial cell Akt15,54,55 and MAPKp42/44.56,57 In all of these endothelial cell studies, similar assays were conducted. Endothelial cells were grown in tissue culture dishes, serum-starved, then soluble Ang1 was added and Akt and/or MAPKp42/44 phosphorylations were assessed. To further compare the effects of soluble Ang1 on myocytes versus endothelial cells, we also conducted this type of assay. We found that Ang1 phosphorylated Akt (Figure 11) and MAPKp42/44 (Figure 12) on C2C12 myocytes, HSMs, and Ms1 endothelial cells. Wortmannin, a phosphatidylinositol 3′-kinase (PI3-K) inhibitor, effectively blocked Akt (Figure 11) and MAPKp42/44 (Figure 12) activation in both myocytes and endothelial cells. Studies have also shown that wortmannin blocks MAPKp42/44 phosphorylation in myoblasts.58 Thus, Ang1-induced myocyte Akt and MAPKp42/44 activation appear to be PI3-K–mediated. We found that Ms1 endothelial cell, but not myocyte, Akt (Figure 11) and MAPKp42/44 (Figure 12) were phosphorylated by 3.6 nmol/L Ang1, a commonly used dose in endothelial cell studies.15,54–57 Ang1 (200 nmol/L) increased phosphorylation of Akt HSM (4.2-fold), C2C12 myocytes (2.4-fold), and Ms1 endothelial cells (2.1-fold). For MAPKp42/44, Ang1 (200 nmol/L) increased HSMs (3.5-fold), C2C12 myocytes (2.3-fold), and Ms1 (3.8-fold).

Discussion

In this study, we show that skeletal and cardiac myocytes adhere to Ang1 and Ang2 via integrins and that Ang1 promotes survival under adverse conditions. This finding revises the current view that angiopoietins act essentially on the vasculature via the endothelial cell (EC)–specific receptor, tie2. With the recognition that angiopoietins act on CMs, ECs, and fibroblasts,32 an expanded role for these molecules in cardiac health and disease may emerge. Angiopoietins appear well-positioned to mediate myocardial remodeling, which requires coordination among these various cell types for proper cardiac function. Interest in mechanisms that regulate balanced remodeling is increasing with the appreciation that disproportionate changes in these cellular components occur in pathological cardiac remodeling and may ultimately contribute to heart failure. Furthermore, our findings indicate that this novel angiopoietin role extends beyond the heart. Cytoprotective effects on skeletal myocytes (HSM, C2C12 myocytes) suggest that Ang1 may also directly
mediate skeletal muscle survival during insults such as limb ischemia from peripheral vascular disease. We propose that this new angiopoietin function acts in concert with effects on the vasculature to protect the heart and peripheral tissues under adverse conditions.

We found that RGD-based peptides and EDTA blocked myocyte adhesion to Ang1 and Ang2 (Figures 2 and 3). Fibroblast adhesion to Ang1 and Ang2, and EC adhesion to Angptl3 are also inhibited by RGD-based peptides and EDTA. These features support integrin involvement. However, Ang1, Ang2, and Angptl3 lack an RGD site. We examined mouse, rat, and human Ang1 and Ang2, and found no exact matches to 22 well-known integrin-binding motifs. However, there were areas resembling integrin motifs in the fibrinogen-like (fbg-lk) domain of Ang1 and Ang2 that were conserved among mouse, rat, human, and other species (eg, QHREDGS). This region resembles KRLDGS (fibrinogen) or REDV (fibronectin) integrin motifs. A QHREDGS or similar amino acid (AA) sequence exists in human Ang1 and 2, mouse Ang1 to 3 and 5, pig Ang1 and 2, chicken Ang2 isoforms A and B, zebrafish Ang1 to 3, Caenorhabditis elegans angiopoietin (Ang)-related protein, human Angptl1 to 6, mouse Angptl3, and yellow fever mosquito Ang-related protein. Mouse Angptl4 and African clawed frog Ang-related protein have a nearby RGD site. An exception is human Ang4, which lacks the D, and has a diverging function from its counterpart. We propose that select myocyte integrins adhere to Ang1 and Ang2 via the fbg-lk domain, possibly at or including the AA’s QHREDGS. In support of this concept, ECs adhere to via /H9251

Figure 6. Ang1 phosphorylates Akt and MAPKp42/44. In C2C12 myocytes, C2C12 myocytes were held in suspension (Sus.) or plated on immobilized 200 mmol/L Ang1, Ang2, laminin (Lam), collagen I (Coll. I), collagen III (Coll. III), collagen IV (Coll. IV), fibrinogen (FN), vitronectin (VN), BSA, or unmodified tissue culture wells (plate) for 40 minutes (n=3/group per study; studies done in duplicate). Akt, phospho-Akt\textsubscript{Ser}473 (A), MAPKp42/44, phospho-MAPKp42/44\textsubscript{Thr}202/204 (B), and FAK and phospho-FAK\textsubscript{Y}397 (C) were measured by Western blotting. Ang1 induced Akt and MAPKp42/44 phosphorylation, but not FAK. *Ang1-induced Akt and MAPKp42/44 activations were significantly greater than all other conditions tested (*P<0.0001).
Our studies implicate integrins (e.g., \(\alpha_6\beta_1\), \(\alpha_5\beta_1\), \(\alpha_4\beta_1\), \(\alpha_3\beta_1\), \(\alpha_5\beta_1\), \(\alpha_6\beta_1\)) in mediating myocyte adhesion to Ang1 and/or Ang2. Our findings together with those of Carlson et al. (fibroblasts)\(^3^2\) suggest that each cell type has a characteristic set of integrins that engages the angiopoietins, which may partially overlap among cell types. Our most complete analysis was conducted on skeletal myocytes (HSM) owing to the greater availability of anti-human integrin antibodies. There are fewer available anti-rat antibodies, so additional integrins may yet be identified that mediate cardiac myocyte adhesion to Ang1 and Ang2. Furthermore, the expression of many integrin subunits shifts during myocyte differentiation.\(^6^3\) This may account for our finding that C2C12 differentiation into myocytes is vital for attachment to Ang1 and Ang2.

Promising integrin subunit candidates include \(\beta_3\) and \(\alpha_6\), \(\alpha_7\). Antibodies blocked mouse, rat, and human myocyte adhesion to Ang1 and Ang2 (Figure 3A through 3C) and prevented Ang1 induction of Akt and MAPK\(_{42/44}\) phosphorylations (Figure 9E and 9F). \(\beta_3\) is important in CM focal adhesion complexes and is active in cardiac hypertrophy.\(^6^4\) Also, \(\alpha_\text{a}\) inhibited myocyte attachment to Ang1 and Ang2 (Figure 3A and 3C) and abrogated MAPK\(_{42/44}\) activation (Figure 9F). \(\alpha_\text{a}\) is vital to CM function, prevalent on CM, and is not expressed by cardiac fibroblasts.\(^6^5\) Isoforms (\(\alpha_\text{aA}\) and \(\alpha_\text{aB}\)) are expressed in fetal and adult hearts,\(^6^6\) and levels shift during cardiac development.\(^6^6,6^7\) The blocking effects of \(\beta_3\) and \(\alpha_6\) antibodies support a link between myocyte-Ang1 interactions and activation of cell signaling (Figures 6 through 12).

We examined the impact of Ang1 on signaling pathways critical to CM survival and protection from apoptosis. Akt phosphorylation is necessary and sufficient for CM cytoprotection. Constitutively active Akt reduced infarct size (64%), CM apoptosis (84%),\(^6^8\) and heart failure\(^6^9\) in vivo. In vitro Akt phosphorylation prevented DNA fragmentation,\(^7^0\) caspase activation, cytochrome c release,\(^7^1\) and CM apoptosis.\(^7^0\) Phosphorylated MAPK\(_{42/44}\) activated CM prosurvival signals\(^7^2\) and reduced reperfusion injury,\(^7^3\) \(\beta\)-adrenergic stimulation\(^3^8\)-induced CM apoptosis, and heart failure.\(^7^4\)
Thus, Ang1 activation of human, mouse, and rat myocyte Akt and MAPKp42/44 signals may increase myocyte survival and prevent apoptosis.

A direct role for Ang1 in preventing caspase-3 activation in CM may ultimately advance efforts toward preserving cardiac function. In a mouse model of ischemia/reperfusion injury, cardiосpecific caspase-3 overexpression increased CM apoptosis, poly-ADP ribose polymerase (DNA repair enzyme) degradation, DNA fragmentation, and infarct size.\(^{75}\) Alternatively, inhibiting caspase-3 prevented CM apoptosis\(^ {76}\) and reduced ischemia injury,\(^ {77,78}\) infarct size,\(^ {79}\) postinfarct remodeling,\(^ {80}\) and heart failure.\(^ {81}\) Activated caspase-3 can degrade CM contractile proteins (myosin light chain-1,\(^ {82}\) sarcomeres,\(^ {83}\) and myofibrils)\(^ {75}\) without inciting cell death.

This contributes to LV remodeling, dilation,\(^ {84}\) and reduced LV function in ischemia,\(^ {84}\) reperfusion injury,\(^ {75}\) infarct, and heart failure.\(^ {82}\)

Our data show that Ang1 and Ang2 are not EC-specific and can use non-tie2 receptors. Ang1 and Ang2 can act directly on cardiac and skeletal myocytes through integrins and promote myocyte survival (Table, Figure 4) through specific pathways such as caspase-3 inhibition (Figure 5) and Akt and MAPKp42/44 phosphorylation (Figures 6 through 12). These actions are similar to those reported for Ang1 as an EC survival factor,\(^ {15,16,54,56,85}\) namely inhibition of caspase,\(^ {54,55}\) and activation of Akt,\(^ {15,54,56}\) MAPKp42/44,\(^ {55}\) PI-3K, survivin, and eNOS.\(^ {86,87}\)

Figure 8. Ang1 phosphorylates Akt and MAPKp42/44 in NCMs. A through C, NCMs were held in suspension (Sus.) or plated on immobilized 200 nmol/L Ang1, Ang2, laminin (Lam), collagen I (Col. I), collagen III (Col. III), collagen IV (Col. IV), fibronectin (FN), vitronectin (VN), BSA, or unmodified tissue culture wells (plate) for 40 minutes (n=3/group per study; studies done in duplicate). Akt, phospho-AktSer473 (A), MAPKp42/44, phospho-MAPKp42(thr202)/44(tyr204) (B), and FAK and phospho-FAKtyr397 (C) were measured by Western blotting. Ang1 induced Akt and MAPKp42/44 phosphorylation (*P<0.05 for Ang1 vs the other conditions), but not FAK.
explored. Precedents suggest that this may be possible. Hepatic fibrinogen/Ang-related protein, which does not bind tie2, prevents HUVEC apoptosis in serum-free media. Angptl3, which also does not bind tie2, adheres to HMVECs via integrin/αvβ3, activating Akt, MAPKp42/44, and some FAK. HUVEC adhesion to Ang1 and Ang2 activates MAPKp42/44 and FAK, and EDTA- and RGD-based peptides nullify these signals, implicating integrins as mediators of angiopoietin signals on ECs. Thus, in the heart, Ang1 may act on CMs and cardiac ECs to promote survival and maintain cardiovascular health.

Ang1 and Ang2 promoted adhesion of all the myocytes studied (Figure 1). However, only Ang1 increased myocyte survival (Figure 4, Table) and regulated cytoprotective signaling (Figures 5 through 12). The observations that both immobilized (Figures 6 through 9) and soluble (Figures 10 through 12) Ang1, but not Ang2, activated myocyte Akt and MAPKp42/44 signaling within 30 minutes, suggests that this distinction is not due to differences in coating affinities or protein degradation. Furthermore, Ang1-induced signal activation remained intact in suspended myocytes (Figure 10), eliminating differences in cell adhesion as an explanation.

Cell adhesion was not required for this response. The different survival effects of the angiopoietins may relate to the region in the fbg-lk domain of each molecule that we propose mediates adhesion. These regions share 77.8% homology, which is greater overlap than that found in the full-length proteins 62% homology. We are exploring the possibility that this region of Ang2 may resemble Ang1 enough to have a similar binding site conformation, but vary in key residues needed to induce signaling. On endothelium, the mechanism of action of Ang2 is complex. Ang2 is a tie2 antagonist on vascular endothelium, and at high concentrations is a weak agonist. On lymphatic endothelium, Ang2 acts as a tie2 agonist. Similar complexities likely characterize the actions of Ang2 on myocytes via integrins.

Soluble Ang1 added to myocytes and endothelial cells grown in tissue culture plates activated Akt (Figure 11) and MAPKp42/44 (Figure 12). Our study is the first to use human recombinant Ang1 to measure myocyte and endothelial cell signaling. Previous studies used a variant form of Ang1, termed Ang1*, which contains the first 73 amino acids of Ang2 fused to the 77th amino acid of Ang1 with a
cysteine to serine mutation at amino acid 265. Ang1* was engineered because it is easier to produce and purify than Ang1. However, sequences within the first 76 amino acids of Ang1 are critical for multimerization and Tie2 activation, and thus may alter the activities of Ang1.

Our studies show similar effects of human Ang1 on human, mouse, and rat myocytes. Several in vivo studies demonstrate that human Ang1 is effective in other animals. In myocardial infarction models, transfection of human Ang1 plasmids or adenoviral vectors reduced infarct zones in mice and rats, respectively. Plasmid-driven overexpression of human Ang1 reduced hindlimb ischemia in rabbits and gastric ulcers in rats.

Mechanisms-of-action and functional significance of angiopoietin–myocyte interactions in vivo remain to be determined. Various ischemia models have shown a protective benefit from overexpressing Ang1. Ang1 overexpression in the heart resulted in smaller infarct zones and preserved cardiac function following myocardial infarctions. Improved myoblast survival and reduced tissue necrosis with hindlimb ischemia and enhanced survival of muscle and skin flaps have also been reported. These studies showed increases in perfusion and/or capillary density, which were thought to explain the protective effects of Ang1 on the muscle and other tissues. However, our findings suggest that nonvascular mechanisms may also contribute to the superior outcomes. We show that Ang1 directly promotes survival of cardiac and skeletal myocytes (Figure 4, Table), prevents caspase-3 activation (Figure 5), and activates cell survival pathways (Figures 6 through 12). This suggests that Ang1...
directly supports myocyte survival in vivo under adverse conditions (eg, ischemia).

In summary, we demonstrated that angiopoietins directly interact with myocytes via integrins to mediate cell adhesion and survival. It is worth noting that Ang1 activated cytoprotective signaling cascades far more potently than any of the other integrin ligands tested, whether in an immobilized (Figures 6 through 9) or soluble (Figures 10 through 12) form. In fact, when cells were held in suspension and incubated with soluble Ang1, Ang1 was more efficacious at inducing anti-apoptotic pathways in myocytes than in endothelial cells; for which it is a well-recognized survival factor.

![Figure 11](http://circres.ahajournals.org/)

**Figure 11.** Soluble Ang1 activates Akt signaling in myocytes and endothelial cells grown in serum-free media on tissue culture plates. C2C12 myocytes (A), HSMs (B), and Ms1 endothelial cells (C) grown adhered to tissue culture plates were incubated with the indicated concentrations of soluble Ang1, Ang2, laminin (Lam), or PBS for 30 minutes. Some wells were preincubated with 200 nmol/L wortmannin (W) for 2 hours before the addition of soluble factors. Cell lysates were prepared and Western blotting conducted for Akt and phospho-AktS473 (n=3/group per study; studies done in duplicate). Ang1 phosphorylated Akt greater than all the other conditions tested (*P<0.008, C2C12) and (*P<0.001, HSM). For Ms1 endothelial cells, Ang1 significantly phosphorylated Akt (3.6 nmol/L, *P=0.006 vs PBS) and (200 nmol/L, **P=0.0005 vs PBS). For Ms1, 200 nmol/L laminin also activated Akt (#P=0.008 vs PBS). Wortmannin abrogated Ang1-induced Akt phosphorylation for C2C12 myocytes, HSMs, and Ms1 endothelial cells.

![Figure 12](http://circres.ahajournals.org/)

**Figure 12.** Soluble Ang1 activates MAPK\(_{42/44}\) signaling in myocytes and endothelial cells grown in serum-free media on tissue culture plates. C2C12 myocytes (A), HSMs (B), and Ms1 endothelial cells (C) adhered to tissue culture plates were incubated with the indicated concentrations of soluble Ang1, Ang2, laminin (Lam), or PBS for 30 minutes. Some wells were preincubated with 200 nmol/L wortmannin (W) for 2 hours before the addition of soluble factors. Cell lysates were prepared and Western blotting conducted for MAPK\(_{42/44}\) and phospho-MAPK\(_{42/44}\) (phosphoMAPK\(_{42/44}\) (n=3/group per study; studies done in duplicate). Ang1 phosphorylated MAPK\(_{42/44}\) greater than all the other conditions tested (*P<0.004, C2C12) and (*P<0.02, HSM). For Ms1 endothelial cells, Ang1 significantly phosphorylated MAPK\(_{42/44}\) (3.6 nmol/L, *P=0.008 vs PBS) and (200 nmol/L, **P=0.008 vs PBS). Wortmannin abrogated Ang1-induced MAPK\(_{42/44}\) phosphorylation for C2C12 myocytes, HSMs, and Ms1 endothelial cells.
These findings support the concept that Ang1-myocyte interactions are biologically relevant. We propose that this new angiopoietin function acts in concert with effects on the vasculature to protect the heart. Furthermore, understanding the role of Ang1 in CM survival may lead to novel therapies to stabilize and maintain CM number and function after cardiac insults, thereby impeding heart failure.

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Angiopoietin-1 Promotes Cardiac and Skeletal Myocyte Survival Through Integrins
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Expanded Methods

Cardiac Myocyte Isolation

Hearts were harvested using sterile technique and placed in cation-free Hank’s Balanced Salt Solution (HBSS). Ventricles were minced, placed in 0.6 mg/ml trypsin/EDTA (4°C shaker overnight). Supernatants were discarded and tissues were resuspended in low glucose (1.0 g/L) DMEM (LG-DMEM)/10% FCS/1% GPS and agitated (10 min, 37°C). Fragments were digested with 1 mg/ml collagenase type II (Worthington) in HBSS with agitation (30 min, 37°C). Supernatants were collected and put on ice. The collagenase digestion step was repeated with remaining tissue (30 min, 37°C). Supernatants were pooled, centrifuged (5 min), and cell pellets resuspended in LG-DMEM/10% FCS/1% GPS and pre-plated twice (70 min each) to remove fibroblasts. Unattached cells were collected, centrifuged (5 min, 4°C), and resuspended in LG-DMEM/10% FCS/1% GPS. Cells were plated overnight at near confluence to minimize fibroblast growth and promote beating networks. Media was changed to LG-DMEM/2% FCS/1% GPS. Myocytes were used in assays within 0-48 hr.

Cell Adhesion Assay

Wells were coated with human fibronectin (Sigma, diluted in PBS, at room temperature for 45 min at 0–300 nmol/L), human vitronectin (Sigma, diluted in sterile water, at 37°C for 2 hr at 0–300 nmol/L), mouse laminin (Gibco, diluted in HG-DMEM, at 37°C for 2 hr at 0–400 nmol/L), human collagen I (0–300 nmol/L) and collagen III (0–700 nmol/L) (Chemicon, reconstituted in 0.5 mol/L acetic acid, diluted in PBS, at room temperature for 1 hr), and mouse collagen IV (R&D Systems, diluted in sterile water, at room temperature for 2 hr at 0–300 nmol/L).
C2C12 myocytes were detached with trypsin/EDTA, rinsed twice in their growth medium, and three times in serum-free medium (HG-DMEM/0.5% BSA). Cells were counted, plated (50,000 cells/well), and incubated (40 min, 37°C, 5% CO₂). Wells were rinsed three times with PBS. Attached cells were fixed in 10% formalin (Fisher) for 30 min. Photomicrographs were taken on a Nikon Eclipse TE300 inverted microscope. 1% toluidine blue/10% formalin was added to wells (1 hr). Wells were rinsed with double-distilled water four times and air-dried overnight. Stained cells were solubilized with 2% sodium dodecyl sulfate (SDS)/PBS and incubated (15 min, 37°C). Absorances (650 nm) were measured in a microplate reader. Wells were prepared in triplicate and assays were done at least twice.

For assays with rat neonatal cardiac myocytes (NCM) and human primary skeletal myocytes (HSM) similar methods were used with the following changes. NCM were rinsed in LG-DMEM/10% FCS/1% GPS twice, and then three times in serum-free medium (LG-DMEM/0.5% BSA) and plated (120,000 cells/well), and HSM were rinsed in BulletKit Complete medium plus growth factors twice, and then three times in serum-free BulletKit basal medium/0.5% BSA and plated (150,000 cells/well).

To characterize myocyte adhesion to angiopoietins, cells were washed in serum-free medium and incubated (30 min, 37°C, 5% CO₂) with one of the following: 10 mmol/L ethylene diaminetetraacetic acid (EDTA) (Ambion), 10 mmol/L ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA) (Sigma), 500 µg/ml GRGDSP (RGD), 500 µg/ml GRGESP (RGE) (Synpep), 50 µg/ml integrin adhesion-blocking antibodies, or PBS. Myocytes were added to ang1-, ang2-, or BSA-coated wells and adhesion was quantified.

Antibodies for mouse and rat myocytes

α₁ hamster anti-mouse monoclonal antibody clone Ha31/8 (BD Biosciences)
α₄ rat anti-mouse monoclonal antibody clone R1-2 (BD Biosciences)
α₅ rat anti-mouse monoclonal antibody clone 5H10-27/ MFR5 (BD Biosciences)
α₆ rat anti-mouse monoclonal antibody clone NKI-GoH3 (Chemicon)
αᵥ hamster anti-mouse monoclonal antibody clone H9.2B8 (BD Biosciences)
β₁ hamster anti-mouse monoclonal antibody clone Ha2/5 (BD Biosciences)
β₃ hamster anti-mouse monoclonal antibody clone 2C9.G2 (BD Biosciences)

Antibodies for human myocytes (Chemicon)
α₁ mouse anti-human monoclonal antibody, clone FB12
α₂ mouse anti-human monoclonal antibody, clone P1E6
α₃ mouse anti-human monoclonal antibody, clone P1B5
α₄ mouse anti-human monoclonal antibody, clone P1H4
α₅ mouse anti-human monoclonal antibody, clone P1D6
α₆ rat anti-human monoclonal antibody, clone NKI-GoH3
αᵥ mouse anti-human monoclonal antibody, clone M9
β₃ mouse anti-human monoclonal antibody, clone 25E11
β₄ mouse anti-human monoclonal antibody, clone ASC-3
αᵥβ₃ mouse anti-human monoclonal antibody, clone LM609
αᵥβ₅ mouse anti-human monoclonal antibody, clone P1F6
αᵥβ₆ mouse anti-human monoclonal antibody, clone 10D5

Divalent cation adhesion assays were conducted as described¹,² with the following changes. Trypsinized C2C12 myocytes were washed twice in HG-DMEM/0.5% BSA/1 mg/ml soybean trypsin inhibitor (Gibco), once in 2 mmol/L EDTA/PBS, and four times in divalent
cation-free HBSS/0.5% BSA/50 mmol/L Hepes with 2 mmol/L CaCl₂, MgCl₂, MnCl₂ or no divalent cations. BSA-, ang1-, and ang2-coated wells were blocked as described, except that wells were washed once with 2 mmol/L EDTA/PBS and three times with HBSS/0.5% BSA/50 mmol/L Hepes.

Myocyte adhesion is shown as mean absorbance ± standard deviation (SD). Two-sample Student’s t-Tests were conducted with $p < 0.05$ considered significant.

**Cell Survival Assays**

Myocytes were plated (C2C12 at 8,500 cells/well; HSM or NCM at 17,000 cells/well) on wells coated with 200 nmol/L ang1, ang2, laminin, fibronectin, vitronectin, collagen I, -III, or -IV, BSA, or unmodified tissue culture wells (plate) as described in adhesion assay and incubated in serum-free media [C2C12 in HG-DMEM/0.5% BSA; NCM in LG-DMEM/ 0.5% BSA; HSM in Bulletkit Basal Medium/ 0.5% BSA] for 1 day at 37°C, 5% CO₂. In trypan blue assays, cells were trypsinized, stained 1:1 with trypan blue, and the number of white and blue cells was counted. Percent viable myocytes is shown as # white cells / total (white + blue) cells (mean±SD). To assess viability using MTT, media was removed and phenol red free DMEM/0.5% BSA/ 0.5 mg/ml MTT was added (4 hr, 37°C, 5% CO₂). In living cells, active mitochondrial dehydrogenases cleave tetrazolium (colorless) to formazan (purple). Media was removed and dimethylsulfoxide (DMSO) was added to dissolve the formazan (15 min with shaking). Absorbances were measured in a microplate reader at 570 nm with reference at 655 nm. Values were corrected for background absorbance to blank wells with DMEM/BSA/MTT. Cells were counted using a Coulter counter. Each point was measured in triplicate and assays were done at least twice. Percent myocyte survival is shown as
absorbance / total myocytes (mean \( \pm \) SD). Two-sample Student’s \( t \)-Tests were conducted with \( p \leq 0.05 \) considered significant. Photomicrographs were taken with a Leica DM-IL inverted microscope using integrated modulation contrast.

**Caspase Assay**

Myocytes were plated (C2C12 at 17,000 cells/well; HSM or NCM at 42,000 cells/well) on 200 nmol/L ang1, ang2, laminin, fibronectin, vitronectin, collagen I, -III, or –IV, BSA, or plate, in serum-free medium [C2C12 in HG-DMEM/0.5% BSA; NCM in LG-DMEM/0.5% BSA; HSM in Bulletkit Basal Medium/ 0.5% BSA] and incubated (overnight, 37°C, 5% \( \text{CO}_2 \)). Media was removed and apoptosis induced by adding 0.2 \( \mu \text{mol/L} \) Paclitaxel as described\(^3\) to serum-free medium and incubating myocytes (1 day, 37°C, 5% \( \text{CO}_2 \)) (\( n=15 \)/group). To measure caspase-3 activity, twelve wells from each group were pooled in pairs to generate \( n=6 \)/group. Myocytes were rinsed (ice-cold PBS), lysed, frozen and thawed (-80°C 15 min; room temperature 10 min; ice 15 min; repeat -80°C and room temperature incubations). Lysates were centrifuged (15,000 x \( g \), 20 min, 4°C) and supernatants collected. Using a 96-well plate, wells containing cell extracts and blanks (no cell extract) were incubated with assay buffer/2% DMSO/10 mmol/L dithiothreitol/0.2 mmol/L DEVD-para-nitroaniline substrate (overnight, 37°C, 5% \( \text{CO}_2 \)). To detect caspase-3 activity, absorbances were measured (405 nm). The remaining wells were trypsinized and counted in triplicate using a Coulter counter. Caspase-3 activity/total myocytes is shown (mean \( \pm \) SD). Two-sample Student’s \( t \)-Tests were conducted with \( p \leq 0.05 \) considered significant.
Cell Signaling

Myocytes added to Immobilized Molecules

Myocytes were prepared and plated (as described, adhesion assay) on immobilized 200 nmol/L ang1, ang2, laminin, fibronectin, vitronectin, collagen I, -III, and –IV, BSA, and plate (unmodified tissue culture wells) in serum-free medium [C2C12 in HG-DMEM/0.5% BSA; NCM in LG-DMEM/ 0.5% BSA; HSM in Bulletkit Basal Medium/ 0.5% BSA] and incubated (30 min, 37°C, 5% CO₂). As a negative control, myocytes were also held in suspension. Myocytes were prepared for signaling analysis as described⁴ with some modifications. Plates were placed on ice, unattached cells were removed and centrifuged. Attached cells were lysed [RIPA buffer (50 mmol/L Tris pH7.4, 1% IGEPAL/CA-630, 0.25% deoxycholate, 150 mmol/L NaCl, 1 mmol/L EDTA/ phenylmethylsulfonylfluoride (PMSF) / Na₃VO₄, 1 ug/ml aprotinin/ leupeptin/ pepstatin, 100 mmol/L NaF] and unattached cell pellets were lysed and combined with the relevant lysates. Lysates were incubated (4°C, 20 min), and Sample Loading Buffer/Reducing Agent (Biorad) added, and samples heated (95°C, 5 min). Proteins were loaded onto 10% Bis-Tris Criterion gels (Biorad), and separated by SDS–polyacrylamide gel electrophoresis (PAGE) and transferred (Trans-Blot Semi-Dry Electrophoresis) to nitrocellulose. Membranes were washed in tris-buffered saline (TBS), pH 7.6 for 5 min and blocked in 5% nonfat dry milk (NFDM) in TBS with 0.1% Tween-20 (TBST) for 1 hr, washed in TBST (3x, 5 min), and primary antibody added at 1:1000 in either TBST/5% BSA for phospho-Akt serine 473 (pAktS473), phospho-MAPK p42/44 threonine 202/tyrosine 204 (pMAPK p42/44T202/Y204) (Cell Signaling), and phospho-FAK tyrosine 397 (pFAKY397) (BD Pharmingen) or TBST/5% NFDM for akt (Cell Signaling), MAPK (1:3000) (Chemicon), and FAK (BD Pharmingen) at 4°C overnight. Membranes were washed (3x, 5
min) in TBST, the appropriate HRP-linked anti-rabbit or anti-mouse secondary antibody (1:2000) in 5% NFDM/TBST added (1 hr), and washed (3x, 5 min) in TBST. Membranes were developed using Enhanced ChemiLuminescent kits (Amersham) and X-OMAT film (Kodak). Each point was run in triplicate and experiments were done in duplicate. For some studies, C2C12 myocytes were prepared in serum-free medium and then, pre-incubated with 10 mmol/L EDTA, 500 µg/ml GGRDSP or GRGESP peptides, 50 µg/ml integrin adhesion-blocking antibodies (anti-α4, anti-α6, anti-β3), or PBS (30 min, 37°C, 5% CO2). Myocytes were also held in suspension for negative controls. Then, these samples were placed on immobilized 200 nmol/L ang1 or vitronectin (30 min, 37°C, 5% CO2) and processed for Western blotting with antibodies for akt, MAPK, pAkt, and pMAPK as described.

**Suspended Cells added to Soluble Molecules**

C2C12 myocytes and Ms1 endothelial cells were prepared (as described, adhesion assay) and incubated (200,000 cells/Eppendorf tube) in serum-free medium [C2C12 in HG-DMEM/0.5% BSA, Ms1 in LG-DMEM/0.5% BSA] with 200 nmol/L ang1, ang2, laminin, fibronectin, vitronectin, or PBS and incubated (30 min, 37°C, 5% CO2). Then, cells were centrifuged, put on ice, supernatants removed, RIPA lysis buffer added, samples processed, and Western Blotting conducted as described.

**Cells Grown on Tissue Culture Plates with Soluble Molecules Added**

C2C12 myocytes, HSM, and Ms1 endothelial cells were cultured in wells of 96-well tissue culture plates in their respective normal growth media. As cells approached confluency, they were rinsed twice [C2C12 in HG-DMEM, Ms1 in LG-DMEM, HSM in Bulletkit Basal Medium],
and placed in this serum-free media for 24 hrs. Wortmannin (200 nmol/L; Sigma) was added to select wells for 2 hr. Then, we added soluble ang1, ang2, laminin, or PBS for 30 min., put plates on ice, lysed cells (RIPA lysis buffer/ Biorad sample-loading buffer/Biorad reducing agent), heated samples (95°C, 5 min), and conducted Western Blotting as described for akt, phospho-akt, MAPK_p42/44, phospho-MAPK_p42/44. Each condition was done in triplicate and experiments performed in duplicate.

Online Data Supplement References

